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Premutation for the Martin-Bell Syndrome Analyzed in a Large Sardinian Family: III. Molecular Analysis With the StB12.3 Probe

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This report complements a series of clinical, cytogenetical, and psychological studies previously reported on a large Sardinian pedigree segregating for premutations and full mutations associated with the Martin-Bell syndrome (MBS). Using the StB12.3 probe, we report now the molecular classification of all of the critical members of the pedigree. These molecular findings are evaluated against the variable phenotypic manifestations of the disease in the course of a six-generation segregation of an MBS premutation allegedly present in a common female progenitor of 14 MBS male patients and 9 female MBS heterozygotes seen in the last two generations. The nature and stepwise progression of MBS-premutations toward the fully manifested Martin-Bell syndrome and the possibility of reverse mutational events toward the normal allele are discussed with respect to the application of the presently available diagnostic tools in genetic counselling.

KEY WORDS: fragile-X syndrome, premutation, Martin-Bell syndrome

INTRODUCTION

In the two preceeding reports of this series [Filippi et al., 1991; Cianchetti et al., 1992], we described a large Sardinian family in which the fully manifest Martin-Bell syndrome (MBS), observed among 14 males of the last generations, is clearly the result of step-wise mutational events occurring along the X-chromosome pathway linking all of them to a common ancestress who must have been heterozygous for an MBS premutation. On the basis of clinical, cytogenetical, neuropsychological, and behavior studies carried out on the relevant members of this pedigree, we thought to have found evidence of heterozygosity for the fully expressed MBS mutation in four daughters of normal transmitting males. To critically evaluate such a finding, whose confirmation would have been of major significance for understanding the mechanism of transition from the MBS premutations to the fully expressed MBS mutations, we have now reclassified all critical members of the pedigree with probe StB12.3. This probe allows a direct molecular identification of all possible genotypes thus far known at the MBS locus [Oberlé et al., 1991; Yu et al., 1991].

The new data reported here are fully compatible with the current knowledge that MBS premutations consist of an amplification of a heritable unstable trinucleotide $(CGG)_n$ repeat [Fu et al., 1991; Kremer et al., 1991] varying from 60 to about 200 copies, whereas the fully expressed MBS mutations are associated with larger amplifications of the same repeat, usually greater than 600 copies and invariably accompanied by hypermethylation of . . . [Oberlé et al., 1991; Yu et al., 1992]. In the light of the new analysis the above mentioned four alleged heterozygotes for MBS, born to normal transmitting males, have been reclassified as heterozygotes for the premutation. Accordingly, the transition from this status to that of a fully expressed MBS mutation has been recognised to occur exclusively during the oogenesis of segregational heterozygotes for the premutation.

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MATERIAL AND METHODS

Data

The 69 DNA samples which are the subject of this study were derived from the critical members of the large MBS Sardinian pedigree described in our two preceeding reports [Filippi et al., 1991; Cianchetti et al., 1992] where full information can be found on genealogical, clinical, and genetical data whose most relevant manifestations are reported in the Results section of the present report alongside with the new information.

Experimental Protocols

Preparations of high molecular weight genomic DNA samples were made by standard procedures [Maniatis et al., 1982] from peripheral white blood cells or from permanent cultures of EBV-transformed B-lymphocytes, kindly established for us by Dr. M. Rocchi and colleagues. The molecular classification of the various MBS genotypes was carried out as described by Rousseau et al. [1991] with the use of probe StB12.3, which allows fairly accurate estimates of the degree of amplification present at the 5′ end of the FMR-1 gene. Individual DNAs were double digested with EcoRI and EagI and the resulting fragments separated by electrophoresis on 0.8% agarose gel followed by transfer on Hybond-N filters (Amersham, Milan, Italy). The filters were exposed to the labeled StB12.3 probe in a hybridization mixture containing 40% formamide and the unbound label was removed with two consecutive washes in 0.5 × SSC and 0.1% SDS at 60°C [Oberlé et al., 1991].

RESULTS AND DISCUSSION

Figure 1 reports an updated drawing of the pedigree with the new notation describing the correct genotype classification derived from the present molecular studies. The classification of phenotypes was performed, according to Oberlé et al. [1991] at the CGG repeat site for the 69 members of the pedigree who agreed to participate in this additional study. They include 14 MBS patients, 8 FRAXA-positive females, 17 FRAXA-negative males, and 30 FRAXA-negative females. We reported the status of the CGG repeat by indicating as "normal" (N) a CGG repeat number below 50 copies, MBS "premutation" (PM), an amplification between 60 and 200 copies, and MBS "full mutation" (F), the one exceeding the 200 copies and associated with hypermethylation of the nearby CpG island. The most relevant clinical and cytogenetical data have been described in our previous publications on the same pedigree [Filippi et al., 1991; Cianchetti et al., 1992]. The conclusions to be drawn from these additional studies are that i) all 14 males classified as MBS patients on clinical and/or cytogenetical ground exhibit the expected molecular phenotype compatible with an amplification of the unstable CGG repeat greater than 600 copies (full mutation), even when the percent of the FRAXA (+) marker was in the borderline range (V-14, V-21, V-36, and V-100); however, one of them (V-100) was found to be mosaic with one band greater than 200 bp and another one greater than 2300 bp; ii) seven out of the eight FRAXA (+) females who had been previously classified as heterozygous for the full mutation [Filippi et al., 1991] are indeed so also at the molecular level, quite independently of their mental capacity; however, individual IV-14 turned out to be heterozygous for the premutation and not for the full mutation as we had previously hypothesized on the basis of the FRAXA phenotype whose percent (2.65) was found to be just above the cutting threshold of 2% which in our study separates the FRAXA (−) from the FRAXA (+) women [Filippi et al., 1991]; ii) with exception of the case just mentioned, all heterozygous carriers of the MBS premutation were found to be FRAXA-negative and mentally normal. This was the case also for individual V-71 who is the only male of the pedigree directly proven to be hemizygous for an MBS premutation. As expected, all the males hemizygous for the MBS mutation and seven out of the nine women heterozygous for it were found to be FRAXA-positive.

The two full mutation heterozygotes who are FRAXA-negative (V-8 and V-12) are also those with the lowest degree of amplification and one of them (V-12) has a clear incomplete methylation of the nearby EagI restriction site shown by the smear present between 3.4 and 3.7 kb. The latter finding is in agreement with the hypothesis that the degree of amplification of the trinucleotide repeat may be one of the major factors influencing the expression of the FRAXA marker [Rousseau et al., 1994].

From the pedigree of Figure 1, redrawn with the information related to the directly assessed presence of the MBS premutation and/or full mutation, it is now possible to say that it took an average of four generations of meioses for the premutation, allegedly present in progenitor I-2, to turn into the full mutations observed in generations V and VI which are all characterized by an amplification of the $p(CGG)_n$ trinucleotide repeat well beyond the threshold 800 copies.

Though we have no direct information on individual I-2, the pedigree evidence suggests that she might have inherited herself the MBS premutation and, consequently, that the latter might actually have gone through a much greater number of generations of meioses before creating medical problems. The impact of this situation on genetic counselling is hard to assess because all the family data thus far available are unfortunately biased for having been collected through an MBS propositus or a close relative of MBS patients, as was the case with our pedigree discovered through a worried pregnant young mother (V-93). We are convinced that the answer to such an important question can only come from extensive population studies on the frequency of MBS premutation status. If the silent mutation in question is proven to have a ubiquitous high-frequency distribution among healthy individuals even in populations with no record of the fully manifested MBS, genetic counselling to carriers of the MBS premutation should be reconsidered.

Finally, we wish to draw attention to the offspring of the three sisters IV-19, IV-23, and IV-26 who are double heterozygotes in repulsion, for the MBS premutation and for the G6PD mediterranean mutant ($Gd^{mediterranean}$) which is highly prevalent in Sardinia [Siniscalco et al.,

1966]. Taken at their face value, individuals V-50 and V-55 should be recombinants and probably also V-61 since she is likely to have received both mutant alleles from her mother in view of the fact that her father is from Northern Italy where the $Gd^{mediterranean}$ mutant is practically absent. Following a recent report on the occurrence of reverse mutations in fragile-X syndrome [Zhong et al., 1993] we are presently investigating the possibility that the normal sons born to the repulsion heterozygotes IV-19 and IV-23 could be instances of reversion from the premutation to the normal allele status.

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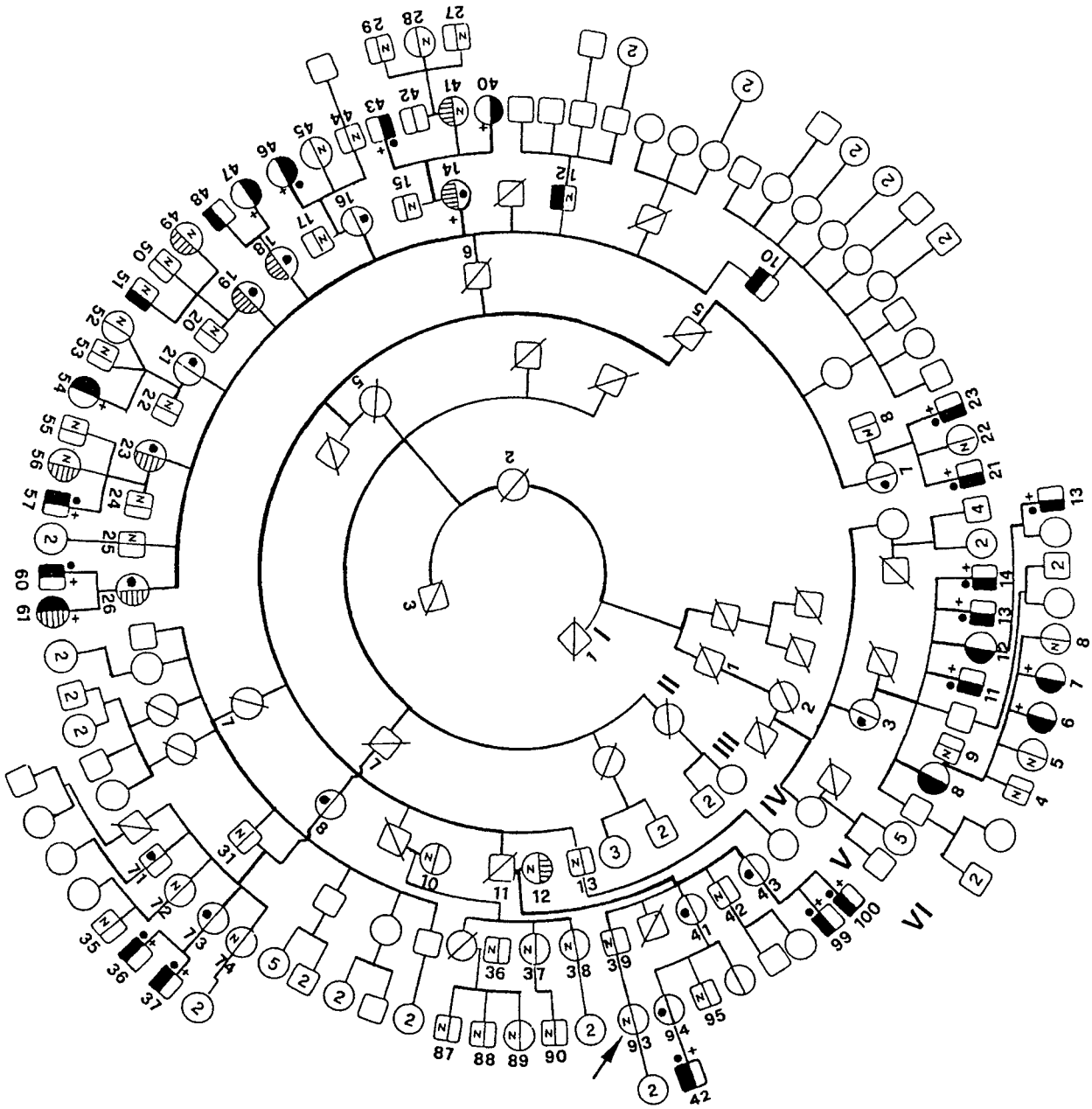

Fig. 1. ⊘ Deceased and Untested; ○ Untested; ◨ ◖ Hemi- or homozygous for normal "MBS allele"; ◨ ◔ Hemi- or heterozygous for "MBS permutation"; ◧ ◐ Hemi- or heterozygous for "MBS full mutation"; ◫ ◑ Hemi- or homozygous for "Gd^{B} allele"; ◨ ◑ Hemi- or homozygous for "$Gd^{mediterranean\ mutant}$"; ◍ Heterozygous $Gd^{B}/Gd^{mediterranean}$. A dot on the upper left side of the symbol means "retarded"; a (+) on the upper right side of the symbol means "FRAXA (+)."

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